

# Structure of the female reproductive system of the lac insect, *Kerria chinensis* (Sternorrhyncha, Coccoidea: Kerridae)

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## Abstract

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The ovaries of female lac insects, *Kerria chinensis* Mahd (Sternorrhyncha: Coccoidea: Kerridae), at the last nymphal stage are composed of several balloon-like clusters of cystocytes with different sizes. Each cluster consists of several clusters of cystocytes arranging in rosette forms. At the adult stage, the pair of ovaries consists of about 600 ovarioles of the telotrophic-meroistic type. An unusual feature when considering most scale insects is that the lateral oviducts are highly branched, each with a number of short ovarioles. Each ovariole is subdivided into an anterior trophic chamber (trophiarium) containing six or seven large trophocytes and a posterior vitellarium harbouring one oocyte which is connected with the trophic chamber via a nutritive cord. No terminal filament is present. Late-stage adult females show synchronized development of the ovarioles, while in undernourished females, a small proportion of ovarioles proceed to maturity.

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## Introduction

Lac insects in the genus *Kerria* (Coccoidea) are cultivated in a region through Asia that includes India, Pakistan, Burma, Thailand and China (Chen *et al.* 2011). In lac insects, masses of sedentary females secrete a protective covering that is periodically harvested and treated to produce valuable natural products including dye, shellac and wax. These products have, to an extent, been displaced by artificial variants but they continue to be used because they are considered safe for human use and environmentally friendly (Lagowska and Golan 2009).

Lac insects are sexually dimorphic (Ross and Shuker 2009). The adult males are very small, about 1 mm long, and are present in two forms, either with or without wings. Mature males have no mouthparts and can live for only 3–4 days during which time they fertilize the females (Marotta 1997). Their total lifespan is about 60 days. Females have a longer lifespan of about 180 days (Subunsenee and Tunthawuthito 1982). Females are immobile and sac-like with significantly reduced wings, legs, eyes and antennae (Miller and Kosztarab 1979; Ben-Dov 1997). The resin encrusts the branches of

trees where large colonies are present. The female *Kerria lacca* appears to be ovoviviparous, because fertilized eggs develop within the female and hatch as nymphs a few hours after oviposition, although some say that they are viviparous (Tremblay 1997; Lagowska and Golan 2009).

The development of the ovary of scale insects is quite different from that of other pterygote insects in that ovarioles protrude from the ovary interior into the body cavity, while in other insects, ovarioles develop inside the ovary (Büning 1994; Szklarzewicz 1997). Diverse patterns of ovariole organization are seen among Hemiptera, all of which have telotrophic-meroistic ovaries (Huebner 1984; Büning 1993, 1994, 2006). The ovarioles of scale insects are distinctive in that they do not possess terminal filaments, they contain a small number of germ cells and follicular cells do not diversify into subpopulations (Szklarzewicz and Bilinski 1995; Szklarzewicz 1997; Szklarzewicz 1998a,b; Koteja *et al.* 2003). The distal trophiarium is composed of a cluster of large trophocytes that connect to the trophic core with a nutritive cord running from the core to the vitellarium with its single oocyte (Szklarzewicz and Bilinski 1995; Szklarzewicz 1997; Szklarzewicz 1998a,b; Koteja *et al.* 2003).

The scale insects are divided into two informal groups based on morphology, anatomy, chromosome behaviour, development and molecular data: the Archaeococcoidea, considered to show many plesiomorphic features, and the Neococcoidea with more apomorphic features (Cook *et al.* 2002; Gullan and Cook 2007; Kondo *et al.* 2008). The family Kerridae to which *Kerria chinensis* belongs lies within the Neococcoidea. Ovariole structure is divergent in these two groups; in particular, in the Archaeococcoidea and just two families of the Neococcoidea, the trophic chamber contains many trophocytes and arrested oocytes, while in the rest of the Neococcoidea, the trophic chamber contains fewer than eight trophocytes and no arrested oocytes (Szklarzewicz and Bilinski 1995; Szklarzewicz 1997; Szklarzewicz 1998a,b; Koteja *et al.* 2003; Szklarzewicz *et al.* 2010).

Although the ovarian structure and development of a number of scale insects have been studied, only one species of lac insect, *Laccifer lacca*, of family Kerridae has been examined in a study that is some 80 years old (Misra 1931). In this study, the ovariole development of *K. chinensis*, another representative of family Kerridae, has been studied using light, electron and confocal microscopy to establish whether it shows the morphological features typical of Neococcoidea. The obtained results may be useful for ongoing taxonomic and phylogenetic studies and increase our knowledge of the reproductive capability of this commercially valuable species.

## Material and Methods

### Insect

*Kerria chinensis* were obtained from Sukhothai Province Forest Products Research station, Royal Forest Department, Sukhothai Province, in the north of Thailand during February to April, 2011–2012. The insects naturally live on stems of rain trees (*Samanea saman*). To assess the effect of nutrient shortage, twigs encrusted with females at the age of about 4 months were cut from the host plants and kept in a 25 °C incubator for approximately 2–3 weeks.

### Light and electron microscopy

Females of 2–5 months old were dissected in phosphate-buffered saline (PBS), and their ovaries were observed using a BX41 Olympus light microscope and photographed with an Olympus DP72 digital camera. For staining with DAPI and rhodamine-phalloidin, the ovaries were fixed with 4% formaldehyde for 30 min, washed with PBS and then incubated in 1 µg/mL DAPI overnight. After washed with PBS, the ovaries were stained with 2 µg/mL rhodamine-phalloidin for 30 min and then washed with PBS. The stained ovaries were observed and imaged using a Nikon C2+ confocal microscope. For sectioning, female insects were fixed over-

night with 2% glutaraldehyde at 4 °C. After washing, the specimens were postfixed in 1% osmium tetroxide for 2 h, washed with PBS and dehydrated in an ethanol series. Specimens were embedded in epon resin prior to sectioning. Semi-thin sections were stained with 1% toluidine blue and observed under a BX41 Olympus light microscope, and all photographs were taken by an Olympus DP72 digital camera.

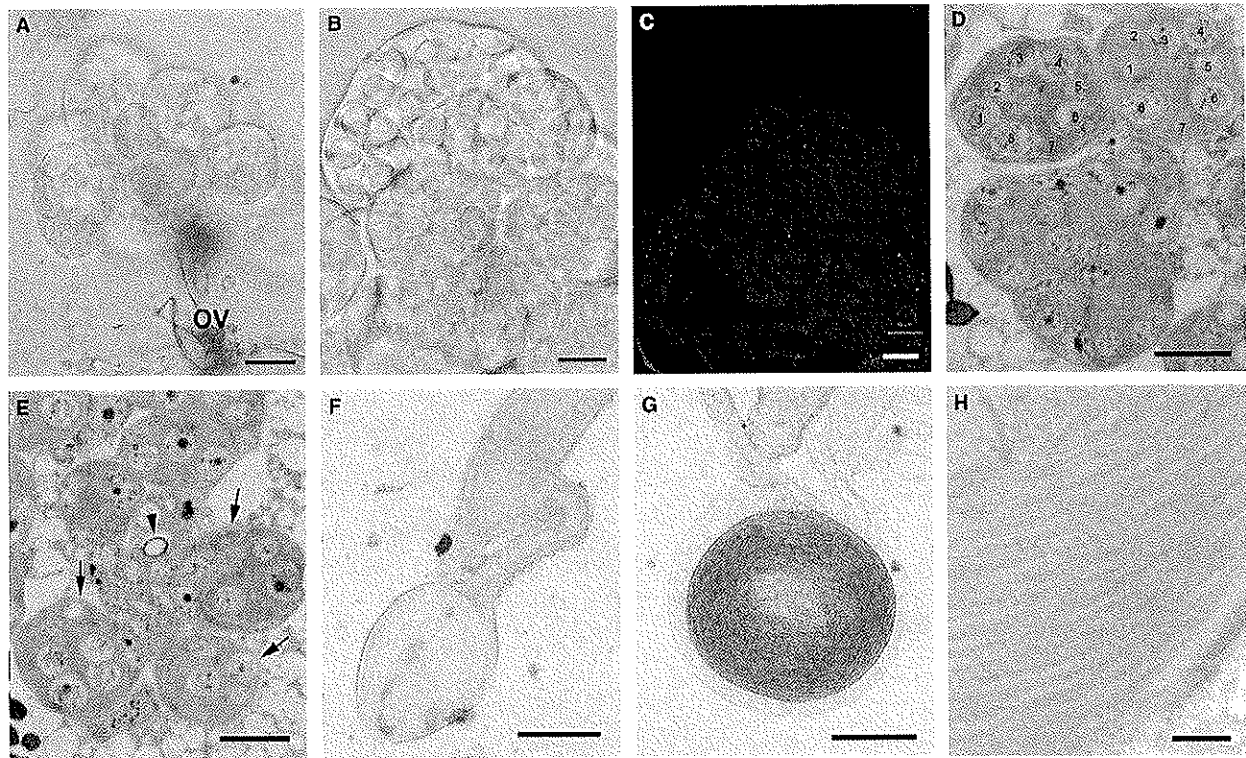
## Results

### The structure of nymphal ovary

During the last nymphal stage (45–50 days after settlement on a twig), the ovary consists of several clusters of cystocytes in the balloon-like shape with different sizes (Fig. 1A,B). At higher magnification, each balloon-like structure can be seen to be composed of several cystocyte clusters (Fig. 1B). Each cone-shaped cystocyte contains a large, spherical nucleus with a relatively round nucleolus (Fig. 1C,D). Using serial semi-thin section, each cluster consists of seven or eight cystocytes appearing in a rosette form (Fig. 1D). Over time, the balloon-like clusters of cystocytes become separated into smaller clusters and finally into individual clusters that join with the branches of the lateral oviducts (Fig. 1E). At this stage, a single rounded spermatheca is quite obvious to be observed (Fig. 1F). The spermatheca of the last nymphal female before being mated is oval, about 200 µm long and 150 µm wide (Fig. 1G), while in mated females at the beginning of early adult stage, the sizes of their spermathecas could enlarge up to 300 µm in diameter depending on the number of deposited sperm bundles (Fig. 1H).

### The general morphology of the ovary of the adult female

Adult female insects (4–5 months old) have a pair of ovaries each composed of about 300 short ovarioles (Fig. 2A). The ovaries are bilaterally paired, connecting to a pair of lateral oviducts that branch from the common oviduct in a Y-shaped pattern (Fig. 2B). The spermatheca attaches to the common oviduct near the junction with the lateral oviducts. Because the sperm bundles are released from the spermatheca to fertilize the ovarioles, the size of spermatheca is smaller than that of the nymphal female (Fig. 2B). Each lateral oviduct divides into several branches each with a radial arrangement of ovarioles towards the apex of each branch (Fig. 2C). In early-stage females, dissected at about 3 months of age, the ovarioles at the apex of each branch are less developed than the basal ones; thus, ovariole development is asynchronous at this stage (Fig. 2D). However, in late-stage females, observed at about 5 months old, almost all ovarioles are at the same, advanced stage of development (Fig. 2E). In females with nutrient shortage, some ovarioles develop to maturity, while the others remain undeveloped as indicated by their relative sizes (Fig. 2F).



**Fig. 1**—The ovary of the last larval instar and young adult female of *Kerria chinensis*. —**A**. The nymphal ovary consists of balloon-like groups of several clusters of cystocytes that are connected with the lateral oviduct (OV), bar = 20  $\mu\text{m}$ . —**B**. Each balloon-like structure is composed of many rosette clusters of cystocytes, bar = 10  $\mu\text{m}$ . —**C**. The DAPI staining shows numerous nuclei of cystocytes in the nymphal ovary, bar = 10  $\mu\text{m}$ . —**D**. A section through the nymphal ovary shows that each cystocyte cluster consists of eight cells, bar = 10  $\mu\text{m}$ . —**E**. Light microscope section indicating several individual nymphal ovarioles. The arrowhead points at the lumen of a branch of the lateral oviduct, bar = 10  $\mu\text{m}$ . —**F**. A spermatheca of late nymphal female before sperm deposition, bar = 100  $\mu\text{m}$ . —**G**. A spermatheca of early adult female containing numerous sperm bundles, bar = 100  $\mu\text{m}$ . —**H**. A spermatheca at higher magnification showing sperm bundles in the spermatheca, bar = 10  $\mu\text{m}$ .

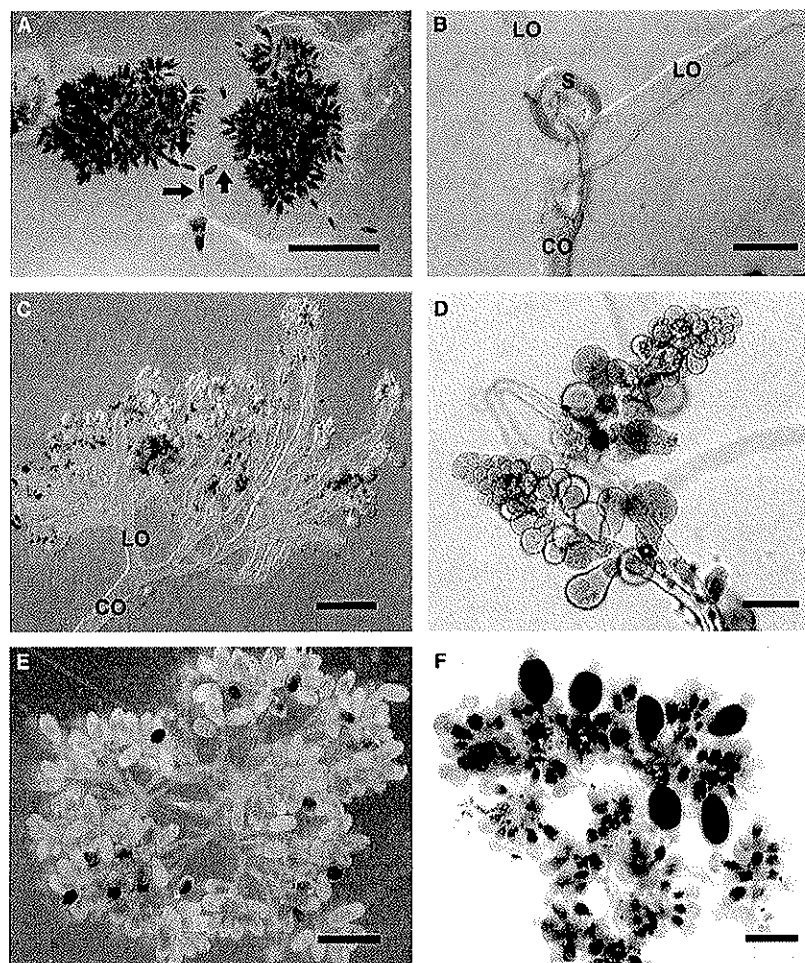
#### The structure of the ovarioles

The ovarioles of *K. chinensis* are of the telotrophic-meroistic type. The young ovarioles are more or less spherical with no terminal filament, and each is attached to a branch of the lateral oviduct via a pedicel (Fig. 3A). In later developmental stages, each ovariole is visibly subdivided into three parts, a distal trophic chamber (tropharium), a proximal vitellarium and a pedicel (Fig. 3A). Initially, the dome-like trophic chamber is larger than the cone-like vitellarium (Fig. 3A,B). The trophic chamber is surrounded by an inner epithelial sheath of relatively flat cells surrounding six or seven large trophocytes (nurse cells), as revealed by confocal microscopy and semi-thin serial sections (Fig. 3C,D,E). The DAPI and rhodamine-phalloidin staining reveal that the trophocytes are cone-shaped and turn their narrow points attaching the centre of the trophic chamber called the trophic core. The nuclei of trophocytes are quite very large and irregular in shape (Fig. 3B,C), each containing a single distinctly spherical nucleolus although in some cells, additional smaller nucleoli are present (Fig. 3D,E). A densely staining, fibrillar nutritive

cord connecting the trophocytes to the vitellarium is visible in sections (Fig. 3F).

Staining with rhodamine-phalloidin also revealed the distribution of F-actin (microfilaments) in the *K. chinensis* ovariole at the middle stage of ovariole development when the trophic chamber is larger than the vitellarium (Fig. 3B, C). In the trophic chamber, the F-actin is located in the cortical cytoplasm of trophocytes and in the trophic core (Fig. 3B,C).

The vitellarium of each ovariole contains only one oocyte with one spherical nucleus (Fig. 4A). The oocyte is surrounded by a single-layered follicular epithelium (Fig. 3B). In the vitellarium, the microfilaments are distributed in the cortical cytoplasm of follicular cells as well as in the oocyte cortex (Fig. 3B). These follicular cells have ovoid or spherical nuclei (Fig. 4B). As ovariole development proceeds, the relative sizes reverse with the vitellaria gradually enlarging, while the trophic chambers reduce in size until they become undetectable (Fig. 4C). The cytoplasm of fully developed, ovoid oocyte is filled with reserve substances (Fig. 4C).



**Fig. 2**—The ovary of the adult female of *Kerria chinensis*. —**A**. The pigmented, paired ovaries (O) showing the common oviduct (horizontal arrow) and the lateral oviducts (vertical arrows), bar = 500  $\mu$ m. —**B**. The spermatheca (S) joins the common oviduct (CO) just proximal to its junction with the lateral oviducts (LO). —**C**. The lateral oviducts (LO) are highly branched, bar = 200  $\mu$ m. —**D**. Ovarioles located along a branch, with the youngest ovarioles located at the apex of each branch and the older ovarioles at the base, as indicated by their size. —**E**. Ovarioles from older females are all at approximately the same, advanced stage of development, bar = 200  $\mu$ m. —**F**. In females exposed to reduced nutrition, some ovarioles show more advanced development, bar = 100  $\mu$ m.

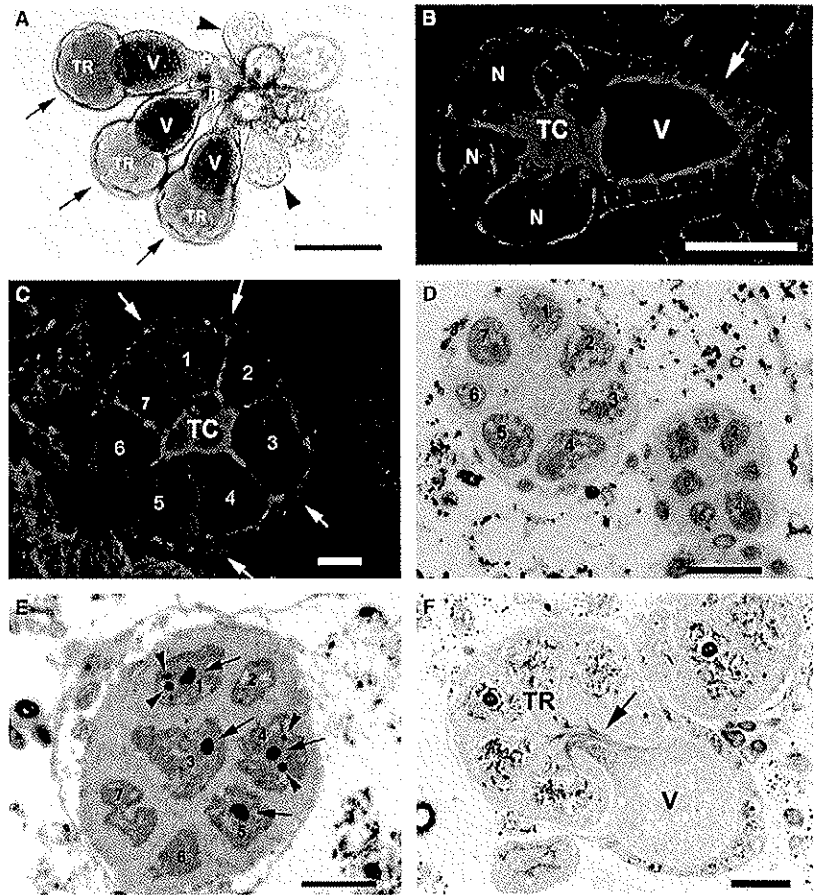
## Discussion

In this study, we describe the ovary structure of female lac insect, *Kerria chinensis*, the commercial lac species of Thailand. A notable feature is that each lateral oviduct divides into several branches, each of which is dotted with many ovarioles as first seen in a study of the related species, *Kerria lacca* (Misra 1931). Therefore, the branched lateral oviduct may be a structural feature common to *Kerria* species. The branched lateral oviducts have been also observed in *Lecanium pomeranicum*, a scale insect belonging to the family Coccidae (Bielenin 1962). By comparison, the lateral oviducts of almost all Coccoidea are unbranched, with the ovarioles dotted along their length. This unbranched arrangement is seen in members of both Archaeococcoidea (Szklarzewicz 1997; Szklarzewicz 1998b; Koteja et al. 2003; Szklarzewicz et al. 2005, 2010) and Neococcoidea (Szklarzewicz 1998a; Ramirez-Cruz et al. 2008). Moreover, the nymphal ovaries of scale insects are usually spindle-shaped (Weglarska 1961; Szklarzewicz 1997), whereas the ovaries dissected from the last nymphal stage of

*K. chinensis* are balloon-like clusters of cystocytes that have not been reported in other scale insects. Because we did not observe the ovaries of younger nymphal stage, it is likely that the ovaries of *K. chinensis* may initially be spindle-shaped before forming balloon-like structures.

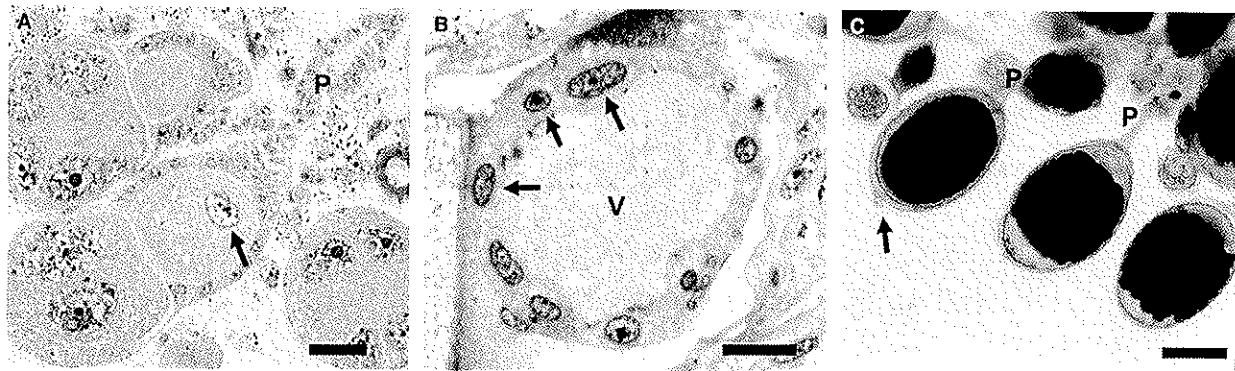
In all scale insects including *K. chinensis*, the larval ovaries consist of cystocytes (germ cells) arranging in a common pattern in which they cluster in rosette forms. The number of cystocytes per cluster varies depending on species: four in *Cryptococcus fagisuga* (Szklarzewicz 1998a), eight in *Nipaecoccus nipae* (Szklarzewicz 1998a) and *Crypticeria morrilli* (Szklarzewicz et al. 2010), 40 in *Puto albicans* (Szklarzewicz et al. 2010), 11–18 in *Porphyrophora polonica* (Szklarzewicz 1998b) and 7–40 in *Orthezia urticae* (Szklarzewicz 1997). In this study, *K. chinensis* has the relatively common pattern of seven or eight cystocytes per cluster. In most insects, a cluster of cystocytes (germ cells) derived from the successive division of a cystoblast, a germ line stem cell. The number of germ cells in a cluster may follow Giardina's  $N = 2^n$  rule ( $N$  is the number of germ cells and  $n$  is the number of successive division of

**Fig. 3**—Ovariolo structure of *Kerria chinensis*. —**A**. Fragment of the ovary of the adult female. Note young spherical ovarioles (arrowheads) and older ovarioles (arrows) subdivided into three parts: a trophic chamber (TR), vitellarium (V) and pedicel (P), bar = 50  $\mu$ m. —**B**. The ovariole stained with DAPI and rhodamine-phalloidin. Note large amount of DNA in the trophocyte nuclei (N) and accumulation of microfilaments in the trophic core (TC) and in the cortical cytoplasm of trophocytes (arrowheads) and follicular cells (arrows), bar = 10  $\mu$ m. —**C**. A single trophic chamber (TC) consists of seven trophocytes. DAPI stains nuclei (numbered) of the trophocytes. Arrows in (C) point at nuclei of inner epithelium sheath, bar = 10  $\mu$ m. —**D**. Transverse sections through trophic chambers show nuclei of seven trophocytes. —**E**. Transverse sections through trophic chambers show nucleoli in seven trophocytes. Each trophocyte has a big spherical (arrows) and some small nucleoli (arrowheads) lie within the nuclei, bar = 20  $\mu$ m. —**F**. Longitudinal section through an ovariole showing the nutritive cord (an arrow) connecting the tropharium (TR) and vitellarium (V), bar = 20  $\mu$ m.



the cystoblasts; Szklarzewicz 1997). As an example, the eight cells per cluster seen in *K. chinensis* could be derived from three successive mitotic divisions of a cystoblast, but seven cystocytes does not fit the pattern. It is possible that seven cystocytes may be generated by three divisions, but one of eight cystocytes may degenerate. Alternatively, one of the cystocytes might not undergo division during the third cycle. However, most members of the Archaeococcoidea do not follow this rule (Szklarzewicz 1998a; Szklarzewicz et al. 2010). In this group, the number of germ cells is relatively high, and in some species, the number can vary within the species, such as 24–44 in *Puto albicans* (Szklarzewicz et al. 2010), 29–58 in *Neuwsteadia floccosa* (Szklarzewicz and Bilinski 1995). It seems that most Neococcoidea including *K. chinensis* obey Giardina's rule; the number of germ cells per ovariole does not exceed eight (Szklarzewicz 1998c). For example, in *Dysmicoccus newsteadii* (family Pseudococcidae), the germ cell number is 8. The smallest number, four germ cells, is found in a number of advanced species such as *Eriococcus buxi*, *Pseudochermes fraxini*, *C. fagisuga* (Szklarzewicz 1998b), *L. pomeranicum* (Bielenin 1962) and *Quadraspidotus ostreaeformis* (Weglarska 1961). However, *Kermes quercus* is the one exception that there are 8–20 cells per cluster (Szklarzewicz 1998b).

The ovaries of *K. chinensis* show a number of features unique to scale insects (Huebner 1984; Büning 1993, 1994; Ramírez-Cruz et al. 2008). The number of ovarioles per *K. chinensis* ovary is about 300 that is similar to the ovaries of scale insects belonging to the Neococcoidea, such as 100–200 reported in *D. newsteadii*, *K. quercus*, *Gossyparia spuria*, *Pseudochermes fraxini* (Szklarzewicz 1998a), approximately 300 in *Eulecanium tiliae* (Koteja et al. 2003) and 400 in *Dactylopius coccis* (Ramírez-Cruz et al. 2008). In most scale insects, the ovarioles develop asynchronously, which prolongs the oviposition period (Koteja et al. 2003). To date, only four species have been reported to possess synchronously developing ovarioles: *Porphyrophora polonica* (Szklarzewicz 1998b), *Steingelia gorodetskia* (Koteja et al. 2003), *P. albicans* and *C. morrilli* (Szklarzewicz et al. 2010). Synchronous development was suggested to indicate an ability to deposit many eggs in a short period (Koteja et al. 2003; Szklarzewicz et al. 2010), which also applies to *K. chinensis*. In a colony of lac insects, the mass emergence of nymphs, called swarming, occurs more or less simultaneously, indicating that a degree of reproductive synchronization occurs across the colony (Subunsenee and Tunthawuttho 1982). We observed that the ovarioles of *K. chinensis* develop asynchronously in early stages but are



**Fig. 4**—The ovarioles of *Kerria chinensis*. —**A**, Longitudinal section through an ovariole showing the pedicel (P). The nucleus of an oocyte is indicated by an arrow, bar = 20  $\mu\text{m}$ . —**B**, Transverse section through a vitellarium (V) surrounded by follicular epithelium cells (arrows indicate nuclei of follicular cells), bar = 20  $\mu\text{m}$ . —**C**, Ovarioles of the older female. An arrow indicates a small, degenerating trophic chamber. P—pedicel, bar = 100  $\mu\text{m}$ .

synchronized in later stages. Because *K. chinensis* females lay only a single batch and then die, it is possible that the ovarioles become quiescent upon maturation, allowing the later-developing ovarioles to catch up to the same stage. In case of nutrient shortage, the ovarioles clearly showed asynchronous development and some ovarioles are fully developed, while the remainders are at various developmental stages. It is possible that synchronous emergence is maintained if the fully developed ovarioles are deposited as eggs and the immature ovarioles regress and die.

The ovarioles of *K. chinensis* are subdivided into three parts, a trophic chamber, vitellarium and a pedicel. The trophic chamber contains six or seven trophocytes (nurse cells), and each vitellarium possesses only one oocyte. The presence of six or seven trophocytes and one oocyte in each ovariole may confirm that they derive from seven or eight cystocytes. Like other insects with meroistic ovarioles, the main function of the trophocytes is to synthesize a number of RNAs and proteins that are transported to the developing oocyte. To increase cell productivity, trophocytes undergo polyploidy, reproducing their chromosomes to many hundred of copies producing large, polyploid nuclei (Gilbert 2010). Consequently, the nuclei of trophocytes are much larger than cystocyte nuclei.

In scale insects, the number of trophocytes per trophic chamber can vary. A large number of trophocytes appear to be the ancestral state; for example, there are 15–35 in *Steingelia gorodetskhia* (Koteja et al. 2003), 17–29 in *N. floccosa* (Szklarzewicz 1997), 23–43 in *P. albicans* (Szklarzewicz et al. 2010). In the derived groups, 3–7 trophocytes are present in each ovariole, with the greatest reduction seen in families Coccididae and Diaspididae, whose trophic chambers contain only three trophocytes (Bielenin 1962; Ksiazkiewicz 1980). The trophic chambers of *K. chinensis* ovarioles contain six or seven large trophocytes and no arrested oocytes as seen in the derived groups, confirming *K. chinensis*' possession of traits

typical of Neococcoidea. All of the features of *K. chinensis* ovaries described in this work – ovarioles with no terminal filaments, only one oocyte in vitellarium, no more than seven trophocytes in a trophic chamber – show as that of scale insect belonging to the advanced group, Neococcoidea.

Looking at the nuclei of *K. chinensis* cystocytes and trophocytes, the nuclei of cystocytes are relatively spherical, whereas the nuclei of trophocytes are quite large and irregular. Moreover, the rhodamine-phalloidin staining of the *K. chinensis* ovarioles revealed concentration of F-actin in the cortical cytoplasm of trophocytes and in the trophic core. The presence of numerous microfilaments in the trophic core and in the trophocytes is connected with the transportation of macromolecules (mainly RNAs) and organelles into the growing oocyte (Gutzeit and Huebner 1986). As in meroistic ovarioles of other insects, the nurse cells of *K. chinensis* ovarioles synthesize large quantities of macromolecules which are transported to the developing oocyte via the trophic core. In *Drosophila melanogaster*, in addition to macromolecules, nurse cell cytoplasm streams via the trophic core into the growing oocyte (Gutzeit 1986). The microfilaments were suggested to be involved in the cytoplasmic streaming process (Gutzeit and Huebner 1986). During the development of *K. chinensis* ovarioles, the large trophic chamber becomes smaller, whereas the vitellarium gradually enlarges. Similar to *D. melanogaster*, the cytoplasm of *K. chinensis* nurse cells may be transported into the oocyte. In the developing oocytes of *K. chinensis*, the concentration of microfilaments in the cortical cytoplasm of follicular cells may reflect the function of F-actin in transportation of vitellogenins from the hemolymph via the follicular epithelium to the developing oocyte (Carcupino et al. 1992; Kaksonen et al. 2006). In the developing oocytes of *K. chinensis*, the presence of F-actin microfilaments found densely on the follicular plasma membrane adjacent to the oocyte may confirm the function of F-actin in the receptor-endocytotic process.

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